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The *Drosophila* Inhibitor of Apoptosis Protein DIAP2 Functions in Innate Immunity and Is Essential To Resist Gram-Negative Bacterial Infection▽

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The founding member of the inhibitor of apoptosis protein (IAP) family was originally identified as a cell death inhibitor. However, recent evidence suggests that IAPs are multifunctional signaling devices that influence diverse biological processes. To investigate the in vivo function of *Drosophila melanogaster* IAP2, we have generated *diap2* null alleles. *diap2* mutant animals develop normally and are fully viable, suggesting that *diap2* is dispensable for proper development. However, these animals were acutely sensitive to infection by gram-negative bacteria. In *Drosophila*, infection by gram-negative bacteria triggers the innate immune response by activating the *immune deficiency* (*imd*) signaling cascade, a NF-κB-dependent pathway that shares striking similarities with the pathway of mammalian tumor necrosis factor receptor 1 (TNFR1). *diap2* mutant flies failed to activate NF-κB-mediated expression of antibacterial peptide genes and, consequently, rapidly succumbed to bacterial infection. Our genetic epistasis analysis places *diap2* downstream of or in parallel to *imd*, *Dredd*, *Tak1*, and *Relish*. Therefore, DIAP2 functions in the host immune response to gram-negative bacteria. In contrast, we find that the *Drosophila* TNFR-associated factor (Traf) family member Traf2 is dispensable in resistance to gram-negative bacterial infection. Taken together, our genetic data identify DIAP2 as an essential component of the Imd signaling cascade, protecting the organism from infiltrating microbes.

Drosophila melanogaster lacks an adaptive immune system and relies exclusively on innate immune reactions for its defense against microbial infection. Activation of the innate immune response leads to the expression of hundreds of genes, some of which encode potent antimicrobial peptides that are synthesized in immunocompetent tissues, such as the tracheal epithelium, circulating "blood" cells, and fat body, the analogue of the mammalian liver (19).

Depending on the infecting microbe, *Drosophila* activates the Toll or *immune deficiency* (Imd) signaling pathway. Exposure to fungi or gram-positive bacteria activates a serine-protease cascade, through pattern recognition molecules (10), that triggers cleavage and activation of Spatzle. Spatzle, in turn, binds and activates the transmembrane Toll receptor, which engages an intracellular signaling cascade that results in nuclear translocation of the NF-κB-like transcription factors Dif and Dorsal. Dif and Dorsal then induce expression of drosomycin, a potent antifungal peptide. In contrast, the Imd pathway is activated in response to gram-negative bacteria. Diaminopimelic acid-type peptidoglycan (DAP-PG), a major component of the gram-negative bacterial cell wall, is recognized by the pattern recognition protein peptidoglycan recognition protein LE (PGRP-LE) and the transmembrane receptor PGRP-LC. Upon binding to DAP-PG, PGRP-LC triggers nuclear translocation of the NF-κB-like transcription factor Relish, which, among others, induces expression of the antibacterial peptide gene *Diptericin* via the Imd pathway (20, 38).

The Imd pathway shares striking similarities with the tumor necrosis factor receptor 1 (TNFR1) signaling cascade (48). Following exposure to pathogens, PGRP-LC activates Imd, which carries a C-terminal death domain that is similar to the domain of the mammalian adaptor protein receptor-interacting protein 1 (RIP1) (11). Through this death domain, Imd recruits dFADD and the *Drosophila* caspase 8 orthologue Dredd to the PGRP-LC receptor (4, 28, 29, 33). Microbe-driven complex formation triggers activation of the *Drosophila* mitogen-activated protein kinase kinase kinase Tak1 and the Relish kinase complex Ird5/Kenny (IκB kinase β [IKKβ]/IKKγ complex) (31, 39, 44, 51). The similarities between Imd and TNFR1 signaling also extend to ubiquitin-mediated activation of IKK (3). As in mammals, *Drosophila* Ubc13(Bendless)/UEV1A, an E2 ubiquitin-protein conjugase complex that promotes K63-linked polyubiquitylation, is required for the activation of Tak1 and the IKK complex (57). Relish activation requires at least two posttranslational modifications, phosphorylation and Dredd-dependent proteolytic cleavage (44–46). These changes enable translocation of Relish to the nucleus and expression of antibacterial peptide genes. However, recent in vivo evidence suggests that Tak1-mediated Jun N-terminal protein kinase (JNK) activation, in addition to Relish activation, is required for antimicrobial peptide gene expression (9). These data are consistent with a model whereby Imd signaling bifurcates at the level of Tak1, which activates both JNK and IKK signaling. Thus, a cooperative input from JNK and NF-κB

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signaling seems to be required for full induction of antibacterial peptide gene expression in response to bacterial infection (see Fig. 8). Loss-of-function mutations in any of the components of the Imd signaling cascade result in the same immune deficiency phenotype, in which animals become acutely susceptible to infection by gram-negative bacteria. Common to all these mutants is their failure to induce expression of antibacterial peptide genes and, therefore, to fend off bacterial infection (38).

Signaling through the mammalian TNFR1 also results in the recruitment of cellular inhibitor of apoptosis protein 1 (c-IAP1) and c-IAP2, two members of the evolutionarily conserved IAP family (37, 41). Although IAPs were originally identified as inhibitor of apoptosis proteins (7), recent evidence suggests that IAPs also fulfill functions that operate independently of their ability to control caspases and cell death (50). Thus, c-IAP1 and c-IAP2, through their ability to bind to TNFR1, TNFR-associated factor 2 (TRAF2), and RIP1, are implicated in modulating TNFR1 signaling. However, due to redundancy or compensatory mechanisms among these IAPs, mutant animals did not display aberrant TNFR1 signaling (5, 6, 42). Recent genome-wide RNA interference (RNAi) screens in cultured cells as well as in vivo RNAi analysis identified the *Drosophila* inhibitor of apoptosis 2 (DiAP2) as a potential component of the *Drosophila* Imd pathway (12, 23). However, due to lack of *diap2* mutant animals, the physiological role of DIAP2 for *Drosophila* immune responses is not fully established.

To investigate the in vivo function of DIAP2, we have generated *diap2* null alleles. *diap2* mutant animals develop normally and are fully viable, suggesting that *diap2* is dispensable for proper development. However, these animals were acutely sensitive to infection by gram-negative bacteria. Consistently, *diap2* mutant flies failed to induce expression of the antibacterial peptide genes and, hence, to mount a proper innate immune response. Thus, our data unambiguously demonstrate that DIAP2 is an essential component of the Imd signaling cascade in vivo, protecting flies from microbial infection.

MATERIALS AND METHODS

Fly stocks. *Oregon*^{R} and *Canton*^{S} flies were used as wild-type controls. *spatzle*^{rm7}, *Tak1*^{1}, *Relish*^{E20}, and *Traf2*^{Ex1} alleles, *Hsp*-GAL4, *Act5c*-GAL4, *Da*-GAL4 drivers, upstream activation sequence (UAS)-*imd*, *hsp*-GAL4, UAS-*Tak1*, UAS-*Relish* (full-length) and UAS-*Dredd* transgenic flies were described previously (2, 18, 29, 51). The *EP*(*G2326*) line was purchased from Genexel Inc. (Daejeon, South Korea). *Df(2R)exel7138* spans the *diap2* locus (35). *diap2*^{7c} and *diap2*^{7a} alleles were generated by transposase-mediated imprecise excision of *EP*(*G2326*) and mapped as indicated below. The UAS-*diap2* construct was generated by cloning the *diap2* open reading frame in EcoRI/XhoI-digested pUAST vector. *w*^{1118} flies were used to generate UAS-*diap2* transgenic flies. An insertion of this construct on the third chromosome was used in this study. *Drosophila* stocks and crosses were maintained at 25°C. Following septic injury, flies were incubated at either 25°C for quantitative reverse transcription-PCR (RT-PCR) analysis or 29°C for survival assays. Heat shock-mediated induction of *imd*, *Tak1*, *Relish*, and *Dredd* overexpression was performed at 37°C for 1 h, followed by a recovery phase at 25°C.

Analysis of genomic lesions. Genomic DNA from homozygous *diap2*^{7c} and *diap2*^{7a} flies was extracted from an adult individual as described previously (13). Five microliters of genomic DNA was used for PCR amplification using Easy-A High-Fidelity PCR cloning enzyme (Stratagene, United Kingdom). The following oligonucleotide primers were used to amplify the *diap2* locus: 5′-CGGGGC ACATCACTTGAAGACCG-3′ and 5′-GGCATTGCCCATGGGCTTAAGC-3′). The resulting PCR product was purified, cloned into pGEMt vector (Promega), and analyzed by DNA sequencing.

Immunoblot analysis. Protein extracts were prepared from five adults or third-instar larvae by snap-freezing, homogenizing, and boiling in Laemmli buffer. Protein samples were analyzed by immunoblotting with anti-DIAP1 (55), anti-DIAP2, and antitubulin antibodies (Sigma, United Kingdom). Anti-DIAP2 was generated in rabbit using a purified, recombinant DIAP2 fragment spanning the baculovirus IAP repeat 3 (BIR3) region (amino acids 215 to 281). For immunoblot detection and quantification of signals, Odyssey technology was used according to the manufacturer's instructions (Licor Biosciences, United Kingdom).

Bacterial strains, infection experiments, and survival analysis. Microbial septic injuries were performed by pricking third-instar larvae in the posterior region or adults in the lateral part of the thorax with a thin needle previously dipped into a concentrated (optical density of ≈200) culture of *Erwinia carotovora* subsp. *carotovora* 15, *Micrococcus luteus*, *Enterococcus faecalis*, or *Candida albicans*. For natural infection by *Erwinia carotovora* subsp. *carotovora* 15, *Drosophila* third-instar larvae were exposed to a mixture of crushed banana and bacteria as described previously (1). For the survival experiments, flies were examined at different time points to monitor survival after septic injury. The infected flies were transferred to fresh vials daily. The experiments were performed using at least 40 flies for each genotype.

Quantitative real-time PCR. For quantitative analysis of *Attacin-A*, *Cecropin-A1*, *Defensin*, *Diptericin*, *Drosocin*, *Drosomycin*, *Metchnikowin*, and *rp49* mRNA expression, RNA was extracted from whole animals using RNA TRIzol (Invitrogen). cDNAs were synthesized using SuperScript II (Invitrogen) and quantitative PCR was performed using double-stranded DNA dye SYBR green I (Roche Diagnostics). Primer pairs were as follows: for *Attacin-A*, sense, 5′-CCCGGAG TGAAGGATG-3′, antisense, 5′-GTTGCTGTGCGTCAAG-3′; for *Cecropin-A1*, sense, 5′-GAACTTCTACAACATCTTCGT-3′, antisense, 5′TCCCAGTCC CTGGATT-3′; for *Defensin*, sense, 5′-GTTCTTCGTTCTCGTGG-3′, antisense, 5′-CTTTGAACCCCTTGGC-3′; for *Diptericin*, sense, 5′-GCTGCGCAATCGC TTCTACT-3′, antisense, 5′-TGGTGGAGTGGGCTTCATG-3′; for *Drosocin*, sense, 5′-CCATCGTTTTCCTGCT-3′, antisense, 5′-CTTGAGTCAGGTGATC C-3′; for *Drosomycin*, sense, 5′-CGTGAGAACCTTTTCCAATATGATG-3′, antisense, 5′-TCCCAGGACCACCAGCAT-3′; for *Metchnikowin*, sense, 5′-AA CTTAATCTTGGAGCGA-3′, antisense, 5′-CGGTCTTGGTTGGTTAG-3′; and for *rp49*, sense, 5′-GACGCTTCAAGGGACAGTATCTG-3′, antisense, 5′-AAAC GCGGTTCTGCATGAG-3′. SYBR green analysis was performed on a Lightcycler (Roche Diagnostics). The amount of mRNA detected was normalized to control *rp49* mRNA values. We used normalized data to quantify the relative levels of a given mRNA according to cycling threshold analysis.

RESULTS

Generation of *diap2* null mutant flies. To elucidate the in vivo function of DIAP2, we created a null allele of *diap2* by screening for imprecise excisions of an existing P element, *EP*(*G2326*) (Genexel Inc.) (Fig. 1A). *EP*(*G2326*) is inserted at position 11449676 (*Drosophila* genome release 4.2.1), which is located 12 nucleotides (nt) 3′ of the transcriptional start site of the *diap2*-RB transcript, and 69 nt 5′ of the transcriptional start site of the *diap2*-RA transcript. We obtained two mutants, *diap2*^{7a} and *diap2*^{7c}, which carried deletions that removed the start codon and extended 371 nt (*diap2*^{7a}) and 735 nt (*diap2*^{7c}) into the coding region of DIAP2. Thus, *diap2*^{7a} lacks the entire first exon (778-bp genomic deletion, from 11449676 to 11448898), while *diap2*^{7c} lacks exon one and most of exon two (1,357-bp genomic deletion, from 11449676 to 11448319) (Fig. 1A). Immunoblot analysis with an anti-DIAP2 antibody, raised against the BIR3 domain of DIAP2, indicated that DIAP2 protein was not detectable in *diap2*^{7a} and *diap2*^{7c} homozygous, *diap2*^{7a}/*diap2*^{7c} transheterozygous, or *diap2*^{7c}/*Def(2R)exel7138* (*diap2*^{7c}/*def*) hemizygous mutant animals. In contrast, DIAP2 protein was readily detectable in animals of the parental *EP*(*G2326*) line as well as in *diap2*^{7c}/+ or *def*/+ individuals (Fig. 1B and data not shown). Importantly, the deletions in *diap2*^{7a} and *diap2*^{7c} did not affect the transcriptional start site and open reading frame of the nearby gene *CG8297* (Fig. 1A

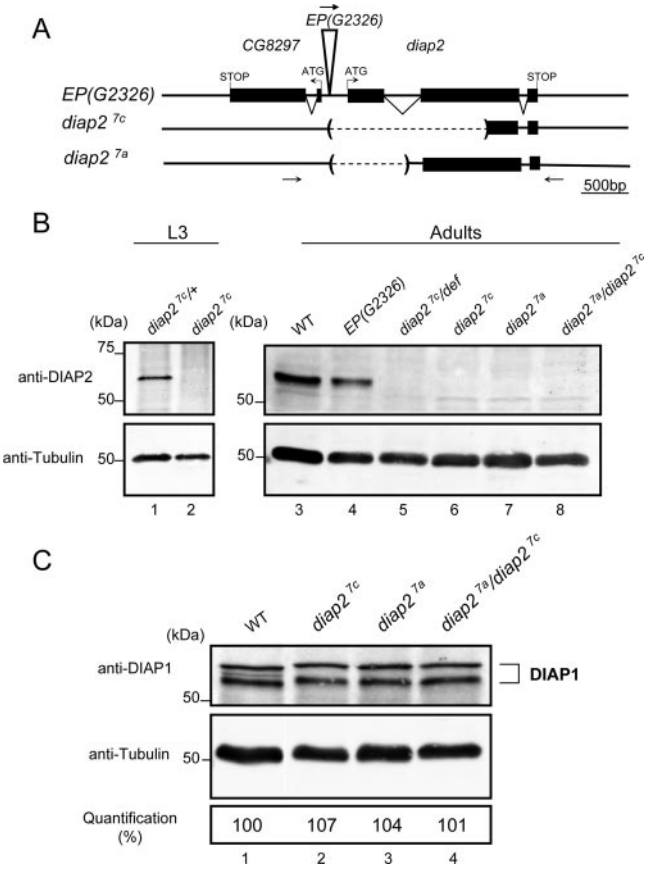

FIG. 1. Generation of DIAP2-deficient flies. (A) Imprecise excision of *EP*(*G2326*) created *diap2*^{7c} and *diap2*^{7a} alleles that carry deletions, which removed large portions of the *diap2* locus. Schematic representation depicting the *diap2* locus, the insertion site and orientation of *EP*(*G2326*), and the genomic DNA. The positions of the primers used to clone the respective genomic DNA fragments are indicated. (B) DIAP2 protein levels were undetectable in *diap2* mutant flies. The presence of DIAP2 protein was examined by immunoblot analysis using anti-DIAP2 antibodies. Protein extracts from the following genotypes were used to monitor DIAP2 expression: *diap2*^{7c}/Cyo-*actGFP* (*diap2*^{7c}/+, third-instar larvae [L3]) (lane 1), *diap2*^{7c} (third-instar larvae) (lane 2), *Canton*^{S} (wild type [WT], adult) (lane 3), *EP*(*G2326*) (adult) (lane 4), *diap2*^{7c}/*Df(2R)exel7138* (*diap2*^{7c}/*def*, adult) (lane 5), *diap2*^{7c} (adult) (lane 6), *diap2*^{7a} (adult) (lane 7), or *diap2*^{7a}/*diap2*^{7c} (adult) (lane 8). Antitubulin immunoblot analysis was used to determine equal protein loading. (C) DIAP1 protein levels remain unchanged in *diap2* mutant flies. The level of DIAP1 protein was examined by immunoblot analysis using anti-DIAP1 and antitubulin antibodies. Quantification of signals was performed using the LICOR system. Protein extracts from the following genotypes were analyzed: *Canton*^{S} (WT, lane 1), *diap2*^{7c} (lane 2), *diap2*^{7a} (lane 3), or *diap2*^{7a}/*diap2*^{7c} (lane 4).

and data not shown). Taken together, these results suggest that *diap2*^{7c} and *diap2*^{7a} are null alleles of *diap2*.

Knockout studies and biochemical characterization of mammalian IAPs have revealed homeostatic cross-regulation among certain IAPs, whereby loss of one IAP can cause compensatory upregulation of family members (6, 15, 42). To address whether loss of DIAP2 resulted in increased levels of DIAP1, we analyzed DIAP1 protein levels in *diap2* mutant flies by quantitative Western blot analysis. As shown in Fig. 1C, DIAP1 levels remained unchanged in *diap2* mutant flies compared to wild-type flies.

Loss of *diap2* renders flies susceptible to septic injury with gram-negative bacteria. Most *diap2*^{7c}/*def* hemizygous mutant individuals survived embryogenesis and developed normally (86% survival, $n = 506$ for *diap2*^{7c}/*def*). Thus, in contrast to *diap1* mutant animals that die early during embryogenesis with deregulated caspase activity (14, 30, 54), loss of zygotic expression of *diap2* did not confer such a phenotype. Although *diap2* mutants showed no obvious developmental defects, we noticed that *diap2* mutant flies were acutely sensitive to infections. To investigate the potential implication of *diap2* in the regulation of *Drosophila* immune response in vivo, we analyzed the survival profile of *diap2* mutant flies in different models of microbial infection by septic injury, an established system to analyze *Drosophila* immune phenotypes (49). To this end, we infected wild-type and mutant flies with the gram-negative bacterium *Erwinia carotovora* subsp. *carotovora* 15 (Fig. 2A), the gram-positive bacterium *Enterococcus faecalis* (Fig. 2B), or the fungus *Candida albicans* (Fig. 2C). Infection by gram-negative bacteria activates the Imd signal transduction pathway, which results in the expression of antibacterial peptide genes. Flies with mutations in *Tak1* (*Tak1*^{1}) and *Relish* (*Relish*^{E20}), two components of the Imd pathway, failed to mount such an Imd response and consequently succumbed to infection by *E. carotovora* subsp. *carotovora* 15 (Fig. 2A) (51). By contrast, a mutation in the *spatzle* gene (*spatzle*^{rm7}), which blocks Toll activation, sensitized animals only to infection by gram-positive bacteria and fungi (Fig. 2B and C) (25, 40). Interestingly, we found that, similar to *Tak1*^{1} and *Relish*^{E20} mutant flies, *diap2* mutant individuals (*diap2*^{7c}/*def*) were highly susceptible to injection of the gram-negative bacterium *E. carotovora* subsp. *carotovora* 15 (Fig. 2A) (51), but not gram-positive bacteria (Fig. 2B) and fungi (Fig. 2C) (36). The survival rate of *diap2*^{7c}/*def* hemizygous mutant animals was almost identical to those of *diap2*^{7c} and *diap2*^{7a} homozygous or *diap2*^{7a}/*diap2*^{7c} transheterozygous mutant animals (Fig. 2A), establishing that *diap2*^{7c} and *diap2*^{7a} are genetically null alleles. Moreover, flies from the parental *EP*(*G2326*) line, which was used to generate the *diap2*^{7c} and *diap2*^{7a} alleles, showed no susceptibility to *E. carotovora* subsp. *carotovora* 15 injection (Fig. 2A). This confirms that the observed immune deficiency phenotype relies on the deletion generated by imprecise excision of *EP*(*G2326*).

Next, we addressed whether the observed susceptibility to *E. carotovora* subsp. *carotovora* 15 infection in *diap2* mutant flies was indeed due to mutation in *diap2*. We took advantage of the yeast UAS/GAL4 binary system to constitutively express a wild-type *diap2* transgene in otherwise *diap2* mutant flies. Expression of the UAS-*diap2* transgene was driven by either the GAL4 driver *Daughterless*-GAL4 (*Da*-GAL4) or *Actin5c*-GAL4 (*Act5c*-GAL4), which express GAL4 constitutively in all cells. Quantitative Western blot analysis was used to determine *diap2* transgene expression levels in otherwise *diap2*^{7c} homozygous mutant flies. While *diap2*^{7c} mutant animals were devoid of DIAP2 protein, "leaky" expression (in the absence of a GAL4 driver) of the *UAS-diap2* transgene resulted in small, but significant, amounts of DIAP2 protein levels (31% of the DIAP2 protein levels observed in wild-type flies [Fig. 3A, lane 4]). In contrast, *Act5c*- or *Da*-GAL4-driven expression of *UAS-diap2* resulted in DIAP2 protein levels that were 64% and 61%

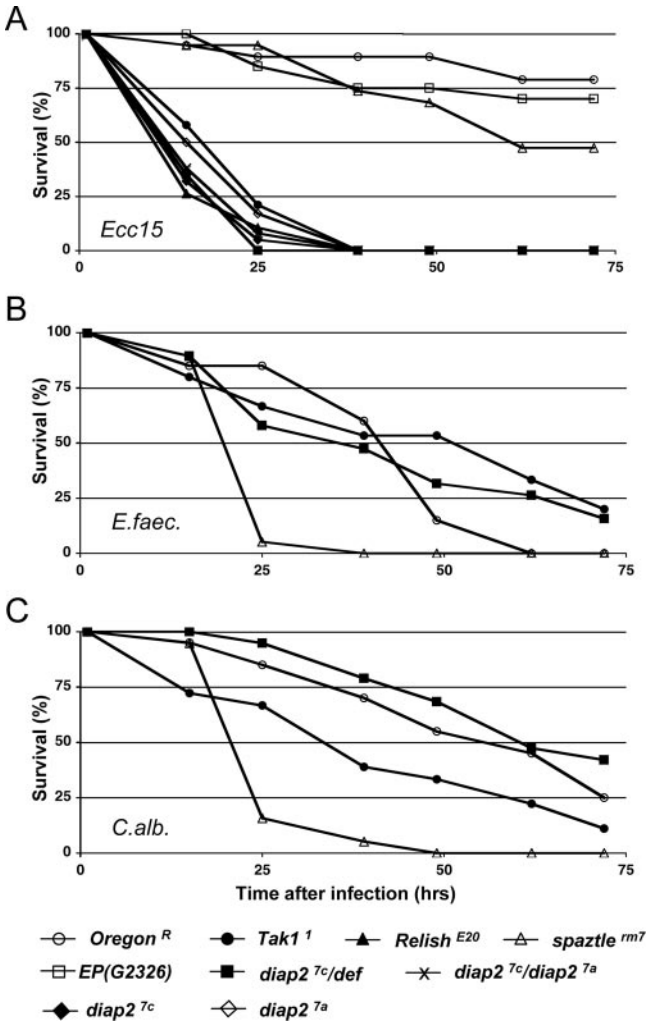

FIG. 2. DIAP2 is required to resist gram-negative bacterial infection. The survival rates of adult males in response to different types of septic injuries are presented. Animals were pricked with a needle previously dipped into *Erwinia carotovora* subsp. *carotovora 15* (*Ecc15*) (A), *Enterococcus faecalis* (*E.faec.*) (B), or *Candida albicans* (*C.alb.*) (C). The following genotypes were examined for susceptibility to microbes: wild-type (*Oregon*^{R}), *Tak1*^{1}, *Relish*^{E20}, *spatzle*^{rm7}, *EP*(*G2326*), *diap2*^{7c}/*def*, *diap2*^{7c}, *diap2*^{7a}, and *diap2*^{7c}/*diap2*^{7a}. Note that *diap2* mutant flies behaved as *Tak1* and *Relish* mutant flies, which are known to be highly susceptible to *Erwinia carotovora* subsp. *carotovora* 15 infection (A), but not to *Enterococcus faecalis* (B) or *Candida albicans* (C) infection. Both *diap2* alleles, *diap2*^{7c} and *diap2*^{7a}, hemizygous (*diap2*^{7c}/*def*) or transheterozygous flies (*diap2*^{7c}/*diap2*^{7a}) showed similar susceptibility to *E. carotovora* subsp. *carotovora* 15 infection, while animals of the parental *EP*(*G2326*) line were fully resistant (A).

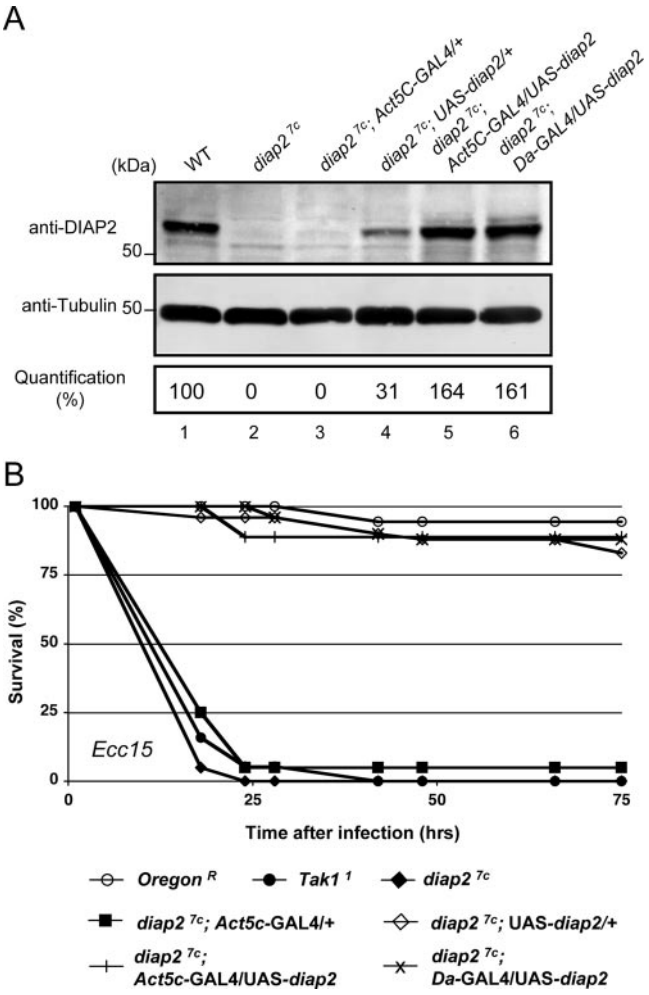

FIG. 3. Ubiquitous expression of DIAP2 fully rescues the immune deficiency phenotype associated with *diap2*^{7c}. (A) Expression level of *diap2* transgene in otherwise *diap2* mutant flies. The presence of DIAP2 protein was examined by immunoblot analysis using anti-DIAP2 and antitubulin antibodies. Protein extracts from the following genotypes were used to monitor DIAP2 protein expression: *Canton*^{S} (wild type [WT]) (lane 1), *diap2*^{7c} (lane 2), *diap2*^{7c}*; Act5C*-GAL4/+ (lane 3), *diap2*^{7c}*; UAS-diap2*/+ (lane 4), *diap2*^{7c}*; UAS-diap2*/*Act5C*-GAL4 (lane 5), and *diap2*^{7c}*; UAS-diap2*/*Da*-GAL4 (lane 6). Quantification of signals was performed using the LICOR system. (B) *diap2* transgene expression rescued *diap2*^{7c} mutant flies from the lethal effects of *Erwinia carotovora* subsp. *carotovora* 15 (*Ecc15*)-mediated septic injury. The following genotypes were examined for susceptibility to microbes: wild type (*Oregon*^{R}), *Tak1*^{1}, *diap2*^{7c}, *diap2*^{7c}*; Act5C*-GAL4/+, *diap2*^{7c}*; UAS-diap2*/+, *diap2*^{7c}*; Act5c*-GAL4/*UAS-diap2*, and *diap2*^{7c}*; Da*-GAL4/*UAS-diap2*.

above wild-type levels (Fig. 3A, lanes 5 and 6). Intriguingly, the "leaky," low level of DIAP2 transgene expression in *diap2*^{7c}*; UAS-diap2*/+ animals was sufficient to completely rescue the immunodeficient phenotype observed in *diap2*^{7c} mutant animals. Even in the absence of GAL4 drivers, *diap2*^{7c}*;UAS-diap2*/+ transgenic flies were fully resistant to infection by gram-negative bacteria and survived microbial exposure like their wild-type counterparts (Fig. 3B). Similarly, *diap2*^{7c} mutant flies with *Act5c*- or *Da*-GAL4-driven expression of *UAS-diap2* were fully resistant to gram-negative bacterial infection, while *diap2*^{7c} flies died within 25 h of septic injury.

Taken together, these data demonstrate that mutations in *diap2* are phenotypically similar to mutations in *Tak1* and *Relish* and that these genes are essential for the resistance to infection by gram-negative bacteria but are dispensable to fend off gram-positive bacterial or fungal infections. Given that *diap2* mutant animals mounted a normal response to infection by gram-positive bacteria and fungi, these data indicate that the immunoresponsive fat body is fully functional in *diap2*

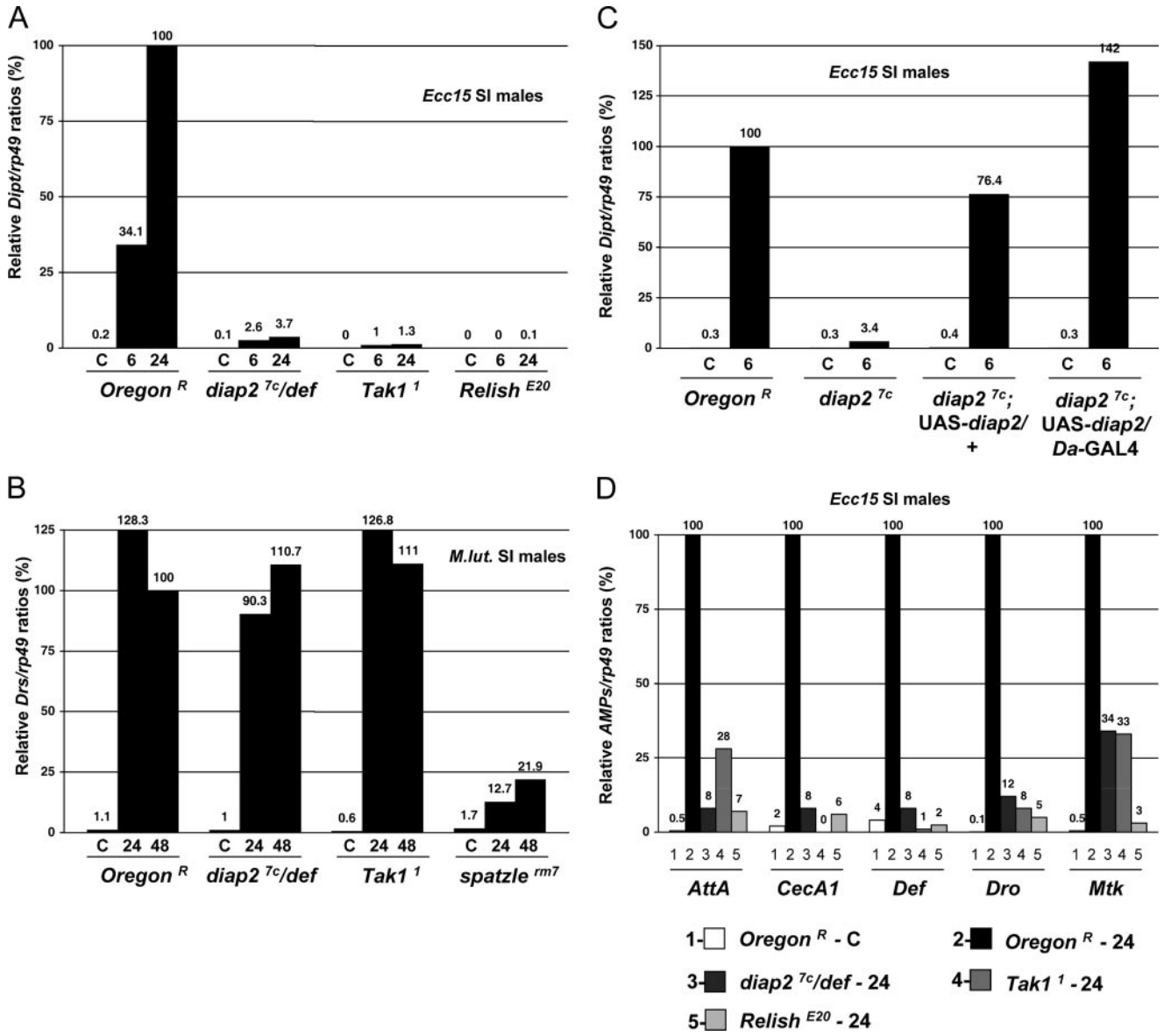

FIG. 4. *diap2* mutant individuals fail to induce antibacterial peptide genes following *Erwinia carotovora* subsp. *carotovora* 15 infection. (A and C) Quantitative RT-PCR analysis of *Diptericin* (*Dipt*) induction after *E. carotovora* subsp. *carotovora* 15 septic injury (*Ecc15* SI) in $diap2^{7c}$, $diap2^{7c}$/*def*, $Tak1^{1}$, and $Relish^{E20}$ mutants, $diap2^{7c}$*; UAS-diap2*/+ and $diap2^{7c}$*; UAS-diap2*/*Da*-GAL4 flies, and wild-type ($Oregon^{R}$) adult males. Results are shown for control (unchallenged) (C) flies and flies 6 and 24 hours after infection. (B) *Drosomycin* (*Drs*) induction after *Micrococcus luteus* septic injury (*M.lut*. SI) of wild-type ($Oregon^{R}$), $diap2^{7c}$/*def*, $Tak1^{1}$, and $spatzle^{rm7}$ adult males. Results are shown for control (unchallenged) (C) flies and flies 24 and 48 hours after infection. (D) *Attacin-A* (*AttA*), *Cecropin-A1* (*CecA1*), *Defensin* (*Def*), *Drosocin* (*Dro*), and *Metchnikowin* (*Mtk*) induction 24 h after *E. carotovora* subsp. *carotovora* 15 septic injury of wild-type ($Oregon^{R}$), $diap2^{7c}$/*def*, $Tak1^{1}$, and $Relish^{E20}$ adult males. Similar to *Tak1* and *Relish* mutants, *diap2* mutant flies were significantly impaired in their ability to induce antibacterial peptide genes in response to gram-negative bacterial septic injury. However, these mutants showed normal *Drosomycin* induction following exposure to gram-positive bacteria. *Diptericin* induction after *E. carotovora* subsp. *carotovora* 15 infection was restored in $diap2^{7c}$ mutant flies expressing the UAS-*diap2* transgene. *rp49* was used as the experimental expression standard. Shown are the relative expression ratios of *Dipt*/*rp49* (A and C), *Drs*/*rp49* (B), *AttA*/*rp49*, *CecA1*/*rp49*, *Def*/*rp49*, *Dro*/*rp49*, and *Mtk*/*rp49* (D).

mutants and that loss of *diap2* does not generally affect fat body development or survival.

DIAP2 is essential for Imd-mediated expression of antibacterial peptide genes. Infection by gram-negative bacteria triggers the Imd signal transduction pathway, which culminates in the transcriptional expression of immune genes, including those encoding antibacterial peptides (8). The expression of one such gene, *Diptericin*, has been established as a reliable and accurate readout to monitor Imd signaling in response to infection by gram-negative bacteria (24). On the other hand, gram-positive bacterial and fungal infections trigger Toll activation and induced expression of *Drosomycin* (25, 40). To test the activation of these pathways in adult *diap2* mutant flies, we monitored *Diptericin* and *Drosomycin* expression by quantita-

tive RT-PCR in response to septic injury with gram-negative (*E. carotovora* subsp. *carotovora* 15 [Fig. 4A]) or gram-positive bacteria (*Micrococcus luteus* [Fig. 4B]). Consistent with the enhanced susceptibility to gram-negative bacteria, we found that *diap2* mutant adult animals displayed a severely compromised immune response to gram-negative bacterial infection. Like *Tak1*^{1} and *Relish*^{E20} mutant flies, *diap2* mutant adult flies failed to induce expression of the *Diptericin* gene following *E. carotovora* subsp. *carotovora* 15 septic injury. A compromised Imd-mediated immune response was already apparent at the larval stage, since *diap2* mutant larvae, like *Tak1*^{1} and *Relish*^{E20} mutants, completely failed to induce *Diptericin* expression upon septic injury (data not shown). In contrast, *diap2* mutant flies were normal in their ability to signal through the Toll pathway. *M. luteus*-mediated induction of *Drosomycin* remained unaffected by the *diap2* mutation. Likewise, *Drosomycin* expression was normal in *Tak1* mutant flies following *M. luteus* septic injury (Fig. 4B). Induced expression of *Drosomycin* in response to gram-positive bacterial infection was abrogated only in *spatzle* mutant flies. Constitutive transgene-mediated expression of *diap2* fully rescued the *diap2*^{7c} mutant immune deficiency phenotype. *diap2*^{7c};UAS-*diap2*/+ flies, like *diap2*^{7c};*Da*-GAL4/UAS-*diap2* or *diap2*^{7c};*Act5c*-GAL4/UAS-*diap2* flies, showed close to wild-type induction of *Diptericin* expression upon *E. carotovora* subsp. *carotovora* 15 infection, while *diap2*^{7c} flies failed to significantly induce *Diptericin* expression (Fig. 4C and data not shown).

To study the requirement of *diap2* for the expression of other antibacterial peptide genes, we assessed the expression of *Attacin-A*, *Cecropin-A1*, *Defensin*, *Drosocin*, and *Metchnikowin*, all of which have been previously reported to be induced in an Imd-dependent manner following gram-negative bacterial infection (26). Following *E. carotovora* subsp. *carotovora* 15 septic injury, expression of *Attacin-A*, *Cecropin-A1*, *Defensin*, *Drosocin*, and *Metchnikowin* were severely impaired in *diap2*^{7c}*/Def*, *Tak1*^{1}, and *Relish*^{E20} mutant flies (Fig. 4D). These results, therefore, indicate that DIAP2 is generally required for activation of Imd-mediated immune responses, while it is dispensable for Toll signaling.

Next, we used an oral infection model to study the effects of the *diap2* mutation. In this model, larvae are naturally infected via the digestive tract through exposure to food contaminated with *E. carotovora* subsp. *carotovora* 15 (Fig. 5) (1). Similar to the results obtained by septic injury, *diap2* mutant larvae that were exposed to *E. carotovora* subsp. *carotovora* 15-contaminated food failed to induce expression of *Diptericin* (Fig. 5A), *Drosocin* (Fig. 5B), or *Attacin-A* (Fig. 5C). *diap2* mutant larvae were similarly immunocompromised as known mutants (*Tak1*^{1} and *Relish*^{E20}) of the Imd pathway. Using a natural model of infection, these data corroborate the notion that DIAP2 is required for Imd-mediated immune response in vivo.

Epistatic positioning of DIAP2. UAS/GAL4-mediated expression of *imd*, *Dredd*, *Tak1*, and *Relish* results in the activation of the Imd signal transduction pathway causing constitutive *Diptericin* expression, even in the absence of infecting microbes (11, 18, 51). We used this system to establish the epistatic position of DIAP2 in the Imd pathway. Heat shock-mediated overexpression of *imd*, *Dredd*, *Tak1*, and *Relish* resulted in induction of *Diptericin* expression (Fig. 6A). The ability to induce *Diptericin* expression varied substantially among *imd*, *Dredd*, *Tak1*, and *Relish*, which is consistent with previous reports (9, 11, 18, 29, 51). Overexpression of *imd* and *Tak1* was most efficient in inducing *Diptericin*, with Imd inducing 63.7% and Tak1 53.4% of *Diptericin* levels observed after septic injury of control animals. In contrast, overexpression of *Dredd* and *Relish* merely achieved 5.9% and 15.7% of normal *Diptericin* expression levels after infection (Fig. 6A). To visualize the contribution of DIAP2 in this system, we set these *Diptericin* expression levels as 100% for all subsequent panels. Intriguingly, in the absence of DIAP2, *imd*-, *Dredd*-, *Tak1*-, and *Relish*-mediated induction of *Diptericin* was blocked (Fig. 6B to

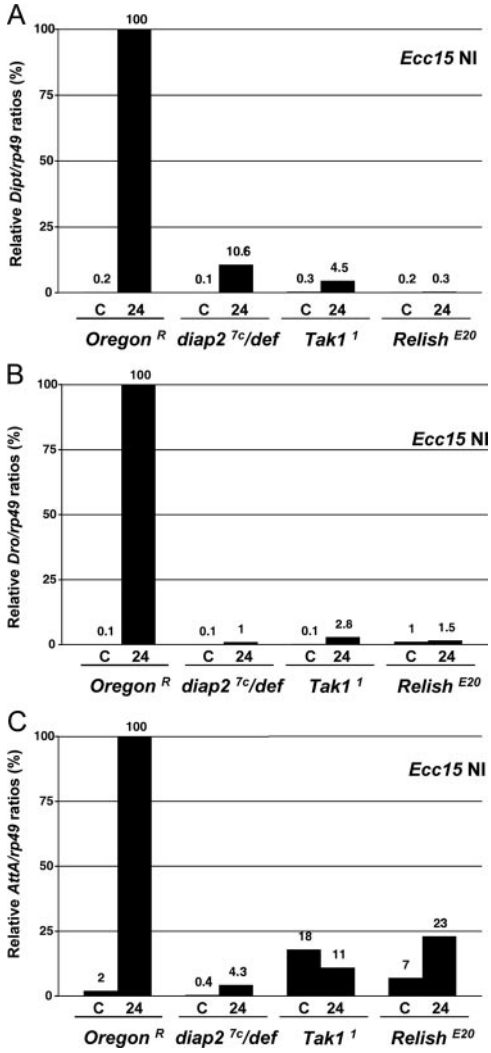

FIG. 5. DIAP2 is required to mount a systemic antibacterial immune response to oral infection by *Erwinia carotovora* subsp. *carotovora* (*Ecc15*). Quantitative RT-PCR analysis of *Diptericin* (*Dipt*) (A), *Drosocin* (*Dro*) (B), and *Attacin-A* (*AttA*) (C) induction after *E. carotovora* subsp. *carotovora* 15 natural infection (*Ecc15* NI) in wild-type (*Oregon*^{R}), *diap2*^{7c}*/def*, *Tak1*^{1}, and *Relish*^{E20} mutant third-instar larvae. Larvae were orally infected by exposing animals to food contaminated with *E. carotovora* subsp. *carotovora* 15. Similar to *Tak1* and *Relish* mutants, *diap2* mutant individuals failed to significantly induce *Attacin-A*, *Diptericin*, and *Drosocin* expression following natural infection. *rp49* was used as an experimental expression standard. The relative *Dipt*/*rp49* (A), *Dro*/*rp49* (B), and *AttA*/*rp49* (C) expression values for control (noninfected) (C) flies and flies 24 h after feeding are shown.

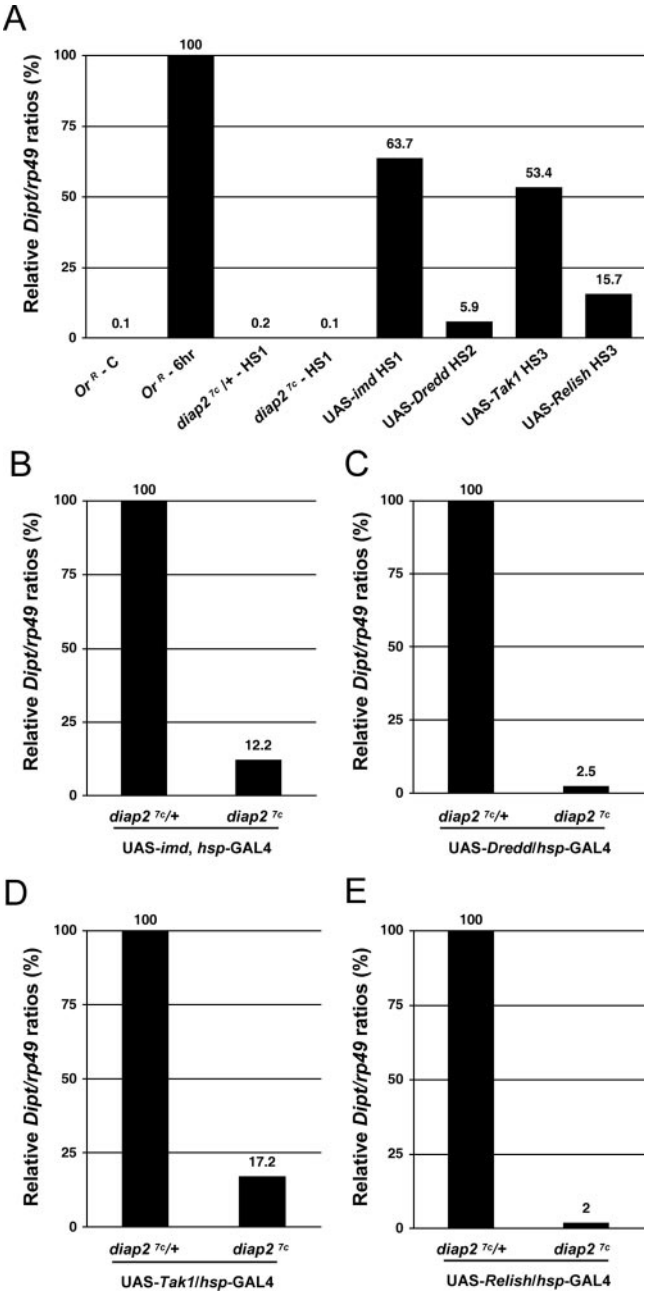

FIG. 6. DIAP2 functions genetically downstream of or in parallel to *imd*, *Dredd*, *Tak1*, and *Relish*. Quantitative RT-PCR analysis of *Diptericin* expression following overexpression of *imd*, *Dredd*, *Tak1*, and *Relish* was performed. (A) *Diptericin* (*Dipt*) expression levels of the following animals are shown: control *Oregon*^{R} animals that were not challenged (Or^R - C) and 6 h after *Erwinia carotovora* subsp. *carotovora* 15 septic injury (Or^R - 6hr); *diap2*^{7c}/+ and *diap2*^{7c} mutant flies 3 h after 1 h of heat shock at 37°C (*diap2*^{7c}/+-HS1 and *diap2*^{7c}-HS1) or heat shock-mediated overexpression of *imd* (UAS-*imd* HS1), *Dredd* (UAS-*Dredd* HS2), *Tak1* (*UAS-Tak1* HS3), and *Relish* (*UAS-Relish* HS3). (B) In the absence of infection, heat shock-mediated *imd* overexpression caused high levels of *Diptericin* (*Dipt*) expression (63.7% of the level of *Diptericin* observed 6 h after *E. carotovora* subsp. *carotovora* 15 septic injury in panel A) that was significantly thwarted in *diap2*^{7c} mutant flies (87.8% reduction). (C) Heat shock-mediated *Dredd* overexpression triggered weak but reproducible *Diptericin* expression (5.9% of the levels of *Diptericin* observed after 6 h of *E. carotovora* subsp. *carotovora* 15 septic injury in panel A), which was blocked in *diap2*^{7c} mutant flies. (D) Heat shock-mediated *Tak1* overexpression triggered strong *Diptericin* expression (53.4% of *Diptericin* levels observed after 6 h of *E. carotovora* subsp. *carotovora* 15 septic injury in panel A) which was blocked in *diap2*^{7c} mutants. (E) Heat shock-mediated *Relish* overexpression triggered weak but reproducible *Diptericin* expression (15.7% of the levels of *Diptericin* observed after 6 h of *E. carotovora* subsp. *carotovora* 15 septic injury in panel A) which was not observed in *diap2*^{7c} mutant flies. Flies were heat shocked for 1 hour at 37°C and left to recover at 25°C for 3 h (A and B) (HS1), 1 h (A and C) (HS2), or 24 h (A, D, and E) (HS3) prior to analysis. The analyzed genotypes were as follows: (i) *diap2*^{7c}/*Cyo*, *act*-GFP (*diap2*^{7c}/+), *diap2*^{7c}; (ii) *diap2*^{7c}/*Cyo*; UAS-*imd*, *hsp*-GAL4/TM6Tb (UAS-*imd*), *diap2*^{7c}; UAS-*imd*, *hsp*-GAL4/TM6Tb; (iii) *diap2*^{7c}/*Cyo*; UAS-*Dredd*/*hsp*-GAL4 (UAS-*Dredd*), *diap2*^{7c}; UAS-*Dredd*/*hsp*-GAL4; (iv) *diap2*^{7c}, UAS-*Tak1*/+; *hsp*-GAL4/+ (UAS-*Tak1*), *diap2*^{7c}, UAS-*Tak1*/*diap2*^{7c}; *hsp*-GAL4/+; (v) *diap2*^{7c}, UAS-*Relish*/+; *hsp*-GAL4/+ (UAS-*Relish*); (vi) *diap2*^{7c}, UAS-*Relish*/*diap2*^{7c}; *hsp*-GAL4/+.

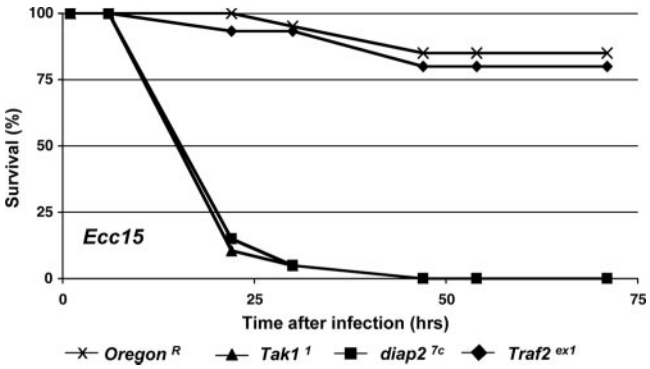

FIG. 7. *Traf2* is dispensable in resisting gram-negative bacterial infection. Shown are the survival rates of *Traf2* null mutant adult males exposed to septic injury with *E. carotovora* subsp. *carotovora* 15 (*Ecc15*). The following genotypes were examined: wild type (*Oregon*^{R}), *Tak1*^{1}, *diap2*^{7c}, and *Traf2*^{ex1}. Note that *Traf2*-deficient flies behaved like wild-type flies, while *diap2* and *Tak1* mutant flies were highly susceptible to *E. carotovora* subsp. *carotovora* 15-mediated septic injury.

E). This indicates that *diap2* is required for *Diptericin* induction by ectopic expression of all these components, corroborating its vital role in Imd signaling. Thus, in this system, *diap2* appears to function downstream of or parallel to *imd*, *Dredd*, *Tak1*, and *Relish*.

***Traf2* is not required for an effective immune response triggered by gram-negative bacterial infection.** In mammals, TRAF2 plays a key role in TNFR1-mediated NF-κB activation (3). Following its recruitment to TNFR1, TRAF2 promotes conjugation of Lys63-linked polyubiquitin chains, which allows the recruitment and activation of Tak1 and IKK (3). Recently, it was suggested that the *Drosophila* Traf2 orthologue contributes to NF-κB-dependent signaling pathways in *Drosophila* (2). To test whether Traf2 is required for Imd-mediated immune responses, we challenged *Traf2* null mutant flies with gram-negative bacteria. Surprisingly, *Traf2* mutant flies were fully resistant to *E. carotovora* subsp. *carotovora* 15 septic injury (Fig. 7). Under the same conditions, *diap2* or *Tak1* mutant animals rapidly succumbed to the same microbial load. Thus, our results demonstrate that *Drosophila Traf2* is dispensable to mount an efficient immune response to infection by gram-negative bacteria.

DISCUSSION

The *Drosophila* innate immune response relies mainly on the differential expression of a variety of small peptides with antimicrobial activities (19). Depending on the infiltrating microbe, *Drosophila* selectively activates two distinct signaling pathways. While infections by fungi or gram-positive bacteria stimulate the Toll pathway, infection by gram-negative bacteria triggers the activation of the *immune deficiency* (Imd) signaling cascade. Activation of both Toll- and Imd signaling results in the activation of NF-κB-like transcription factors leading to the expression of specific sets of antimicrobial peptides (38). Here, we demonstrate through mutation analysis that DIAP2 plays a pivotal role in the *Drosophila* innate immune response. We find that, in vivo, the *Drosophila* inhibitor of apoptosis protein DIAP2 is indispensable for Imd-mediated expression of antibacterial peptide genes. Like known mutants of the Imd pathway, flies with a mutation in the *diap2* gene failed to induce expression of *Attacin-A*, *Cecropin-A1*, *Defensin*, *Diptericin*, *Drosocin*, and *Metchnikowin* and mount an efficient immune reaction in response to infection by gram-negative bacteria. Consequently, *diap2* mutant flies succumbed to gram-negative bacterial infection. In contrast, such flies mounted a normal Toll-dependent immune response and were resistant to infection by fungi and gram-positive bacteria. Our *diap2* null mutant phenotype, therefore, demonstrates that DIAP2 is an essential component of the Imd pathway. Thus, our data are consistent with recent RNAi studies that have implicated *diap2* in the Imd pathway (12, 23).

DIAP2 is a member of the evolutionarily conserved IAP family (17). IAPs are classified by the presence of the BIR domain through which they interact with various "client" proteins (50). Genetic analysis of the *Drosophila* IAP DIAP1 has provided some of the most compelling insights into the in vivo function of this protein family. DIAP1, the first and most extensively studied *Drosophila* IAP, is essential for cell survival and acts as a potent caspase inhibitor (16, 17, 32, 54). Mutations that abrogate physical association of DIAP1 with caspases cause widespread and unrestrained caspase activation, leading to cell and organismal death (14, 30, 54, 55). In contrast to *diap1*, *diap2* null mutants do not show an apparent cell death phenotype and develop normally. This is unexpected, because both these IAPs interact with caspases and IAP antagonists with similar affinities (27, 52, 53). Moreover, when overexpressed, DIAP2 can rescue *diap1* RNAi-mediated apoptosis, suggesting that DIAP2 can functionally substitute for DIAP1 in its ability to regulate caspases (27). Nevertheless, *diap2* mutant animals do not show any apparent apoptosis-related phenotypes during development. However, these animals appear to be sensitized to Reaper-mediated killing in the eye (F. Leulier and P. Meier, unpublished data). The lack of any apparent gross developmental phenotype may be due to sufficiently high levels of DIAP1 that may thwart unscheduled caspase activation in response to loss of DIAP2. In this respect, it is noteworthy that during embryonic development, the levels of *diap1* mRNA dramatically exceed those of *diap2* (17-fold difference, Berkeley Drosophila Genome Project [BDGP] expression profiles). Moreover, similarly to c-IAP2 knockout mice, where a cell death phenotype is revealed only after lipopolysaccharide challenge (5), phenotypic manifestation may become apparent only under certain conditions or in selective tissues. In agreement with this notion, RNAi-mediated depletion of DIAP2 has no effect on cell viability in unchallenged tissue culture cells (12, 23) but significantly sensitizes S2 cells to stress-induced apoptosis (59).

Although IAPs have originally been identified as apoptosis inhibitors (7), recent evidence suggests that IAPs are multifunctional signaling devices that, depending on the protein they interact with, influence diverse biological processes. In this respect, it is noteworthy that IAPs also carry C-terminal RING finger domains providing them with E3 ubiquitin-protein ligase, and hence, signaling activity (50). Thus, in addition to inhibiting apoptosis, IAPs also fulfill functions that operate independently of their ability to control caspases and cell death (50). Therefore, *BIR*-containing proteins are more precisely referred to as BIRCs rather than IAPs (43). Consistent with the notion that BIRCs are multifunctional proteins, the mammalian c-IAP1 and c-IAP2 bind to caspases as well as RIP1 and TRAF2, two components of the TNF receptor signaling complex (34, 37, 41). c-IAP1 or c-IAP2, or both, can promote ubiquitylation and degradation of TRAF2, RIP1, and NF-κB kinase (IKKγ)/NF-κB essential modulator (NEMO) (34, 50). Hence, these BIRC proteins are thought to modulate the response to TNF. More recently, another BIRC protein was identified as an important regulator of innate immune surveillance in mammals. BIRC1e (NAIP5) was found to control the intracellular pathogen *Legionella pneumophila*, a gram-negative microbe that causes severe bacterial pneumonia known as Legionnaires' disease (56). BIRC1e protects infected host macrophages by restricting intracellular replication of this pathogen.

We now find that the *Drosophila* BIRC protein DIAP2 is similarly required for innate immune responses and the resistance to gram-negative bacterial infection. *diap2* null mutants become highly susceptible to gram-negative bacteria and fail to induce antibacterial peptide gene expression. Intriguingly, the Imd pathway, which is required for antibacterial peptide gene expression in response to gram-negative microbes, shares significant similarities with the TNFR1 signaling cascade. The notion that the BIRC proteins c-IAP1, c-IAP2, and DIAP2 are core components of the TNFR1 and Imd pathway, respectively, further reinforces the parallels between the mammalian TNFR1 pathway and the Imd pathway of *Drosophila*, pointing to an evolutionary conservation of these pathways in NF-κB activation (21, 22, 48). Moreover, both pathways seem to rely on ubiquitin-mediated protein modifications. As in human cells, where activation of TAK1 and IKK requires the E2 ubiquitin-conjugating enzyme complex Ubc13/UEV1A (3), *Drosophila* Ubc13(Bendless)/UEV1A are similarly required for activating Tak1 and the *Drosophila* IKK complex (57). Moreover, recent RNAi data from cultured cells suggest that *Drosophila* Tab contributes to Imd signaling, although this still awaits in vivo validation (12, 23, 58). Therefore, similar to the TNFR1 pathway, ubiquitin-mediated protein modification is likely to activate the Tak1/Tab complex via Tab's ability to bind to Lys63-linked polyubiquitin chains, thereby recruiting Tak1 to activator platforms. In contrast to the E2 ubiquitin-conjugating enzyme complex, little is known about the nature of the E3 ubiquitin-ligase of the Imd pathway. While TRAF2 is crucial for Ubc13/UEV1A-mediated ubiquitylation in the mam-

malian TNFR1 pathway, it seems that for Imd signaling Traf2, the TRAF2/6 orthologue in flies, is not a critical component. *Traf2* null mutation did not completely block NF-κB activation in *Drosophila* (2). Moreover, our data clearly indicate that *Traf2* mutant flies are fully competent to mount an immune response and resist gram-negative bacterial infection. Hence, *Traf2* appears not to be essential for an effective Imd-mediated immune response. Since the *Drosophila* genome encodes at least three TRAF family members, it is possible that the loss of Traf2 function is complemented by other TRAF family members. Alternatively, other signaling pathways that bypass Traf2 to transmit the infection signal to NF-κB may exit in *Drosophila*. In agreement with this notion, RNAi-mediated knockdown of all three *Drosophila* TRAFs also did not abrogate Imd-signaling in S2 cells (23, 47, 57). Thus, an E3 ubiquitin ligase different from or in addition to Traf2 may be responsible for Imd signaling in *Drosophila*. Since DIAP2 carries a RING finger domain, it represents a likely candidate.

Our genetic epistatic analysis places *diap2* downstream of or parallel to *imd*, *Dredd*, *Tak1*, and *Relish*. Overexpression of *imd*, *Dredd*, *Tak1*, and *Relish* failed to induce *Diptericin* expression in *diap2* mutant animals, while in wild-type animals, enforced expression of these genes, in the absence of any infection, resulted in reproducible *Diptericin* induction. Intriguingly, *Diptericin* induction following enforced expression of *imd* and *Dredd* is also blocked in *Tak1* mutant animals (9, 29), indicating that both *DIAP2* and *Tak1* are required downstream of *Dredd*. In contrast, *kenny* and *ird5* seem not to be required for *Diptericin* induction when *Dredd* is overexpressed (51). A recent report indicates that Relish cleavage and nuclear translocation on its own are not sufficient for *Diptericin* expression and that, at least in vivo, a further cooperative input from the JNK signaling pathway is required (9). According to this scenario, the Imd signaling pathway bifurcates at the level of *Tak1*, with *Tak1* activating the NF-κB signaling branch as well as JNK signaling branch, both of which are required for expression of antibacterial peptide genes in the fat body. In light of this model, the observation that *diap2* acts genetically downstream of *imd*, *Dredd*, *Tak1*, and *Relish* may indicate that DIAP2 functions at the level of Tak1 (Fig. 8). This view is in agreement with recent reports from *Drosophila* tissue culture cells, which suggest that DIAP2 is required for Tak1-mediated JNK activation (12, 23). In this respect, DIAP2 functions at the same epistatic position as the putative E3 ubiquitin ligase of the Imd pathway. Future biochemical experiments will be required to test whether DIAP2 is indeed the E3 ubiquitin ligase that functions together with Ubc13/UEV1A to stimulate Tak1.

Although the underlying mechanism for the impaired induction of antibacterial peptide gene expression by loss of DIAP2 remains to be defined, the genetic observations made here are likely to have relevance not only for innate immune responses in *Drosophila* but also for TNFR1 signaling in mammals. While in flies DIAP2 is indispensable for Imd signaling, genetic studies in mice have, so far, failed to uncover a physiological role for c-IAP1 and c-IAP2 in TNFR1 signaling (5, 6). Since *c-iap1* knockout mice carry significantly elevated levels of c-IAP2 protein, it is feasible that the increased c-IAP2 levels functionally compensate for the loss of c-IAP1 (6). Consistently, mammalian IAPs have been reported to be under strict homeostatic control by regulating each other's protein levels, which provides a mechanistic explanation for the cross talk among IAPs (42). Thus, in mammals, double-knockout mice lacking both *c-iap1* and *c-iap2* genes will be required to study the roles of c-IAP1 and c-IAP2 in TNFR1 signaling. Therefore, *Drosophila*, where redundancies and compensatory mechanisms are less problematic, provides an ideal model system to study caspase-independent functions of IAPs in an in vivo setting.

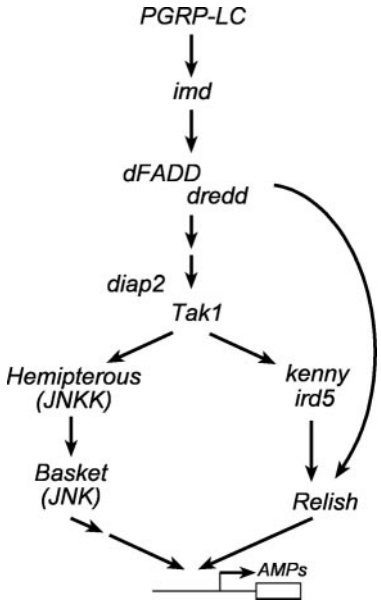

FIG. 8. Model of Imd signaling based on genetic epistasis data in vivo. Our genetic epistasis analysis places *diap2* downstream of or in parallel to *imd*, *Dredd*, *Tak1*, and *Relish*. Intriguingly, loss of *diap2* copies the phenotype of *Tak1* mutant animals, since *Diptericin* induction, following enforced expression of *imd* and *Dredd*, is also blocked in *Tak1* mutant animals, while this is not the case in *kenny* and *ird5* mutant flies. The *Imd* signal transduction pathway bifurcates at the level of *Tak1*, which is required for the activation of the NF-κB signaling branch as well as the JNK signaling branch, both of which are necessary for expression of antibacterial peptide genes in the fat body. We currently favor the model whereby *diap2* functions genetically at the level of *Tak1*. This view is supported by recent reports of *Drosophila* tissue culture cells which suggest that DIAP2 is required for Tak1-mediated JNK activation. Arrows indicate genetic interactions that rely on overexpression of individual components of the Imd pathway in vivo. Note that the ability to induce *Diptericin* expression varied substantially among heat shock-induced overexpression of *imd*, *Dredd*, *Tak1*, and *Relish* (see Fig. 6 for more details). AMPs, antimicrobial peptides.

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